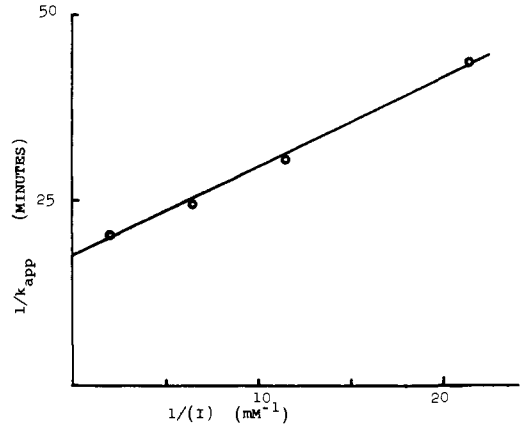

Figure 3. Irreversible inhibition of Ehrlich ascites tumor thymidylate synthetase by 5-iodoacetamidomethyl-2'-deoxyuridine 5'-phosphate (I). Double reciprocal plot of the pseudo-first-order rate constant of inactivation (k_{app}) vs. inhibitor concentration. The values k_3 and K_I were determined from the intercept and slope.

enzyme proceeds through the dissociable complex EI in the equation:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [EI] \xrightarrow{k_3} [EI^*]$$

The model[13] for this type of inhibition has been described for cholinesterase,[14] trypsin,[15] and carboxypeptidase.[16]

A plot of the reciprocal of the pseudo-first-order rate constants ($1/k_{app}$, obtained from the slope of the lines in Figure 2) vs. the reciprocal of the concentration of I (Figure 3) according to the equation

$$1/k_{app} = \frac{K_I}{k_3(I)} + 1/k_3$$

gives a k_3 for the rate of inactivation of 0.057 min^{-1} and K_I of 70 μM which is in reasonable agreement with a K_I of 68 μM for I calculated from the reciprocal plot shown in Figure 1.

References and Notes

(1) B. R. Baker, *Cancer Chemother. Rep.*, **4**, 1 (1959).
(2) Isozymes are defined as structurally different enzymes catalyzing the same transformation. For an extensive discussion of the control of cancer through isozyme specificity refer to B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, N.Y., 1967.
(3) M. Friedkin, *Adv. Enzymol.*, **38**, 235 (1973); P. Reyes and C. Heidelberger, *Mol. Pharmacology*, **1**, 14 (1965).
(4) A. Kampf, C. J. Pillar, W. J. Woodford, and M. P. Mertes, *J. Med. Chem.*, **19**, 909 (1976).
(5) M. Yoshikawa, T. Kato, and T. Takenishi, *Bull. Chem. Soc. Jpn.*, **42**, 3505 (1969).
(6) E. Jenny and D. M. Greenberg, *J. Biol. Chem.*, **238**, 3378 (1963).
(7) A. Fridland, R. J. Langenbach, and C. Heidelberger, *J. Biol. Chem.*, **246**, 7110 (1971).
(8) D. Roberts, *Biochemistry*, **5**, 3546 (1966).
(9) H. Horinishi and D. M. Greenberg, *Biochim. Biophys. Acta*, **258**, 741 (1972).
(10) (a) R. J. Langenbach, P. V. Danenberg, A. Fridland, W. W. Cleland, and C. Heidelberger, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **31**, 419 (1971); (b) P. V. Danenberg, P. J. Langenbach, and C. Heidelberger, *Biochemistry*, **13**, 926 (1974).
(11) The rate of inactivation of the thymus enzyme by compound I (Figure 2) was relatively unchanged over the concentration range of 15–465 μM. This low activity was not a result of decomposition of I in the preincubation media since additions of I at various times during incubation did not alter the rate of inactivation. Furthermore, after incubation of I at 37 °C in the buffer for 6 h, no decomposition could be detected. In contrast after 6 h at 37 °C in 0.5 M KOH, 70% of I remained unchanged.
(12) S. W. Krauss, B. D. Stollar, and M. Friedkin, *J. Virol.*, **11**, 783 (1973).
(13) K. F. Tipton, *Biochem. Pharmacol.*, **22**, 2933 (1973).
(14) R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **237**, 3245 (1962).
(15) E. Shaw and G. Glover, *Arch. Biochim. Biophys.*, **139**, 298 (1970).
(16) G. M. Hass and H. Neurath, *Biochemistry*, **10**, 3535 (1971).

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^{13}C Nuclear Magnetic Resonance Probe of the Active Site of Carbonic Anhydrase

Sir:

We wish to report the successful introduction of a ^{13}C NMR probe into the active site of carbonic anhydrase (carbonate hydrolyase E.C. 4.2.1.1) and its initial use to elucidate important ionization features of the active site. The catalytic activity of this zinc metalloenzyme[1] is known to depend on the ionization of an active site group with p$K_a \sim 7$ whose identity remains in considerable dispute.[1-4] Human carbonic anhydrase B (HCAB) was reacted with 90% [1-^{13}C]bromoacetic acid, an active site directed reagent, under conditions[5] known to lead to the highly specific N$^{\tau}$-monocarboxymethylation of histidine 200 in the amino acid sequence.[6] This active site residue has been found in the crystal structure to lie within a few Angstroms of the essential zinc metal.[7] The carboxymethylated enzyme (CmHCAB) is known to possess its own intrinsic CO_2 hydration[8] and esterase[9] activities that vary between 2.5 and 20% depending on pH and substrate used.

A ^{13}C NMR spectrum of the enzyme modified with enriched bromoacetic acid shows only one peak that is not present in either HCAB or unenriched CmHCAB.[10] This relatively sharp (about 6 Hz line width) resonance is about three times larger than the underlying broad envelope due to the natural abundance signals from the 314 other carbonyl carbons of this 28 900 mol wt enzyme. It is thus due to the *single carbon* of the enriched carboxylate of CmHCAB. In order to test the ability of this potential NMR "probe" to monitor active site events, a titration of the enzyme was carried out while simultaneously measuring the chemical shift of the enriched carboxyl. Figure 1 demonstrates that between pH 5 and 10.5 the probe senses multiple ionizations that must originate in or affect the active site. The smooth curve in Figure 1 is a theoretical fit using a model of two independent ionizing groups with pK_a of 6.0 and 9.2 affecting the resonance.

Reasonable assignments of these pK_a values to active site ionizing groups can be made. The pH 6.0 inflection is almost certainly due to the ionization of the imidazole side chain of

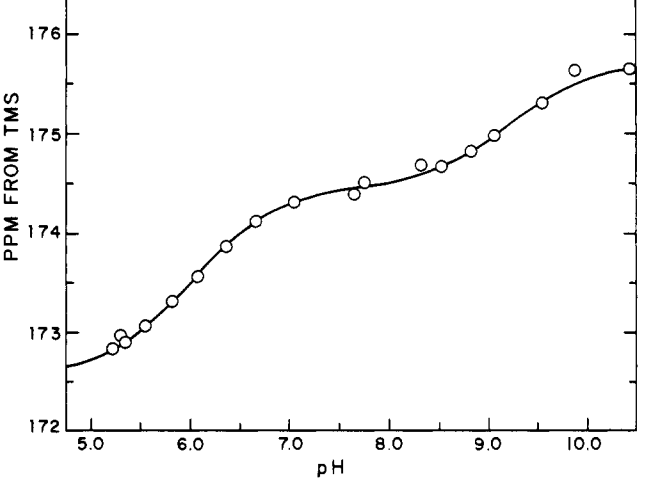

Figure 1. Variation of the chemical shift (Me_4Si scale) of the enriched carboxylate ^{13}C NMR resonance of CmHCAB with pH at 25 °C and 0.2 ionic strength (adjusted with Na_2SO_4 or K_2SO_4). Spectra were obtained at 25.1 MHz on a Jeol PFT-100P/EC 100 FT NMR system using 10-mm sample tubes. Enzyme concentration varied between 1 and 6 mM. All samples contained 10% D_2O for locking and 2-5 μl of dioxane as an internal shift standard (dioxane assumed at 67.40 ppm downfield of Me_4Si). The pH was adjusted by dialysis against buffers and the pH was measured immediately before and immediately after spectra were obtained, rarely changing by more than 0.02 pH units. Smooth curve represents a theoretical fit (see text).

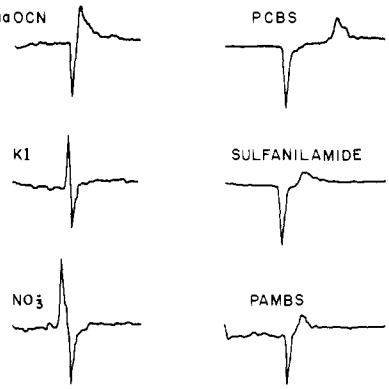

Figure 2. Effects of inhibitors on chemical shift of the ^{13}C NMR resonance of the enriched carboxylate of CmHCAB at pH 7.9 ± 0.1. Each spectrum is the *difference* generated by subtracting the spectrum of the uninhibited enzyme (negative peak marks position of enriched carbon signal) from the spectrum taken in the presence of the indicated inhibitor (positive peak indicates new position of enriched carbon signal). Spectral width shown is 14.2 ppm, negative peak is at 174.54 ppm, and shifts range from a downfield 0.83 ppm for nitrate to an upfield 3.88 ppm for *p*-carboxybenzenesulfonamide (PCBS). PAMBS is *p*-aminomethylbenzenesulfonamide. Spectra are proton decoupled.

histidine 200 (deprotonation of the nitrogen not bearing the carboxymethyl group). The 1.9 ppm downfield shift on deprotonation associated with this ionization is comparable to the 3.1ppm downfield shift observed[10] in the corresponding carboxyl group of N^{τ}-carboxymethyl-L-histidine upon deprotonation of its imidazole ring. The 9.2 inflection is tentatively assigned to the ionization of the zinc-bound water ligand. The downfield shift on deprotonation is consistent with the removal of a positive charge from the vicinity of the carboxylate.[11] Note that there is evidence to rule out a direct inner-sphere coordination of the carboxyl to the metal, at least when Co(II) replaces the zinc.[9] Support for this assignment also comes from observations on the pH dependence of the spectral changes of Co(II) substituted CmHCAB[9] and from pH perturbations of NMR signals of C-2 protons of histidines tentatively assigned to the three zinc ligands in a 270-MHz study[12] of HCAB and CmHCAB. It is worth noting that in this latter study a titrating resonance with pK_a of 6.14 was tentatively assigned to histidine 200 of *native* HCAB. Thus our observations appear to lend support to this assignment of the ^1H NMR study.[13] Our present findings also provide an excellent basis for identifying the group with pK_a of 6.1 recently found by Whitney to influence iodide inhibitor binding in CmHCAB[14] and to affect the visible spectrum of Co^{II}CmHCAB.[9,14]

The probe gives additional promise of providing *microscopic* ionization constants in complexes of the enzyme with inhibitors and substrates. Figure 2 shows that the chemical shift at pH 7.9 is very sensitive to inhibitor binding at the active site. Two main types of carbonic anhydrase inhibitors are represented, the monoanions and the aromatic sulfonamides. Studies are currently in progress to resolve the observed shifts of Figure 2 in terms of contributions from intrinsic changes of the chemical shift of the various ionization states and contributions from alterations of the pK_a values. This and many other aspects of the active site properties are currently being pursued using this promising probe, and attempts are being made to similarly label other histidines in both the B and C isoenzymes using ^{13}C enriched reagents.

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References and Notes

(1) R. G. Khalifah, *J. Biol. Chem.*, **246**, 2561–2573 (1971).
(2) S. Lindskog and J. E. Coleman, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2505–2508 (1973).
(3) Y. Pocker and J. E. Meany, *Biochemistry*, **4**, 2535–2541 (1965).
(4) J. M. Pesando, *Biochemistry*, **14**, 681–688 (1975); D. W. Appleton and B. Sarkar, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1686–1690 (1974); R. B. Martin, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4346–4347 (1974).
(5) P. L. Whitney, P. O. Nyman, and B. G. Malmström, *J. Biol. Chem.*, **242**, 4212–4220 (1967); S. L. Bradbury, *ibid.*, **244**, 2010–2016 (1969); B. Andersson, P. O. Nyman, and L. Strid in "CO_2: Chemical, Biochemical and Physiological Aspects", R. E. Forster, J. T. Edsall, A. B. Otis, and F. J. W. Roughton, Ed., NASA SP-188, Washington, D. C., 1969, pp 109–114.
(6) B. Andersson, P. O. Nyman, and L. Strid, *Biochem. Biophys. Res. Commun.*, **48**, 670–677 (1972).
(7) K. K. Kannan, B. Notstrand, K. Fridborg, S. Lövgren, A. Ohlsson, and M. Petef, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 51–55 (1975).
(8) R. G. Khalifah and J. T. Edsall, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 1986–1989 (1973).
(9) P. L. Whitney, *Eur. J. Biochem.*, **16**, 126–135 (1970).
(10) R. G. Khalifah, D. J. Strader, S. H. Bryant, and S. M. Gibson, manuscript in preparation. The modification of HCAB is carried out near pH 7, at low reagent concentrations (10–20 mM), and for a relatively short time (3–8 h). These conditions have been shown[5] to lead to the complete and exclusive modification of histidine 200. The NMR spectra also show no evidence for side-products.
(11) J. G. Batchelor, *J. Am. Chem. Soc.*, **97**, 3410–3415 (1975); J. G. Batchelor, J. Feeny, and G. C. K. Roberts, *J. Magn. Reson.*, **20**, 19–38 (1975).
(12) I. D. Campbell, S. Lindskog, and A. I. White, *J. Mol. Biol.*, **90**, 469–489 (1974).
(13) This is predicated, however, on the absence of major differences in the properties of histidine 200 in the two enzyme forms. A difference is apparent in our preliminary results on inhibitor binding, where the pK_a of this residue appears far more sensitive to inhibitors than found[12] in *native* HCAB for the ^1H resonance tentatively assigned to histidine 200.
(14) P. L. Whitney and H. Brandt, *J. Biol. Chem.*, in press.

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The Structure of Q* Nucleoside Isolated from Rabbit Liver Transfer Ribonucleic Acid

Sir:

The Q nucleoside which is located in the first position of the anticodon of *E. coli* $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ has been assigned structure **1**;[1,2] the relative stereochemistry of the cyclopentene substituents have been determined as 3,4-trans and 4,5-cis on the basis of NMR comparisons with synthetic models.[3] We have recently shown that the tRNA's of various animals contain a new nucleoside Q*, and that the Q* content is generally more abundant than that of Q.[4] In particular it is to be noted that hepatoma cells have larger quantities of Q* as compared to normal cells.[4] In the following, we show that Q* nucleoside isolated from rabbit liver consists of a mixture of **2** (major) and **3** (minor); it is the first tRNA nucleoside to contain a sugar moiety in the side chain,[5] and is the most structurally complex nucleoside thus far known.